AWARD NUMBER: W81XWH-14-1-0017

TITLE: Biomarkers for Taxane Sensitivity and Hormonal Resistance in Patients with Castration-Resistant Prostate Cancer

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REPORT DATE: April 2016

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
April 2016	Final	1 Feb 2014 - 31 Jan 2016

4. TITLE AND SUBTITLE

Biomarkers for Taxane Sensitivity and Hormonal Resistance in Patients with Castration-Resistant Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-14-1-0017

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This is the final report for a one year Hypothesis Development award initially accepted for funding 1/4/2013. Because of sequestration and delays in approvals between UW and DOD, the award was initiated 2/1/2014. The objective of this project was to utilize the presence of splice variant androgen receptor (AR) in circulating tumor cells (CTC) or disseminated tumor cells (DTC) to predict sensitivity to chemotherapy (docetaxel).

Progress: Aims 1 and 2 are complete. Please see the detailed report for information regarding the presence of new types of AR splice variants detected in metastasis biopsies and the comparisons of detection AR splice variants in CTC, DTC and metastasis biopsies.

15. SUBJECT TERMS

Androgen receptor, splice variant, circulating tumor cells, castration resistant prostate cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	13	19b. TELEPHONE NUMBER *(include area code)*

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1. **INTRODUCTION:** This is the final report for a one year Hypothesis Development award initially accepted for funding 1/4/2013. Because of sequestration and delays in approvals between UW and DOD, the award was initiated 2/1/2014. The original objective of this project was to utilize the presence of splice variant androgen receptor (AR) in circulating tumor cells (CTC) or disseminated tumor cells (DTC) to predict sensitivity to chemotherapy (docetaxel). The focus of the project changed and this change was approved in the annual report due to several factors. 1) A large study assessing the impact of androgen receptor (AR) splice variants on docetaxel sensitivity was published during the project period, making the original focus less significant (Antonarakis et al., 2015) and 2) exciting data regarding the presence and types of AR splice variants in metastatic tissue was developed by our group through the SU2C/PCF metastasis biopsy study discussed in the body of this report. These developments made it more relevant for the focus of the project to change to evaluating the presence of the known and novel AR splice variants from biopsies, disseminated tumor cells (DTC) and circulating tumor cells (CTC) to assist in determining which of these approaches would be sensitive and specific. Assessments of AR splice variants may improve out ability to define mechanisms of resistance to the new generation of hormonal therapies such as abiraterone and enzalutamide for men with prostate cancer.

   The overarching rationale for assessing splice variant AR is that these receptors are isoforms of the wild type AR which lack the ligand binding domain and as a result are autonomously active. They do not require ligand and the ligand binding domain is the site where all of the clinically available AR antagonists bind, making AR splice variants a potential mechanism of resistance to therapies which suppress hormone levels (e.g. abiraterone) or block ligand binding to the AR (e.g. enzalutamide).

2. **KEYWORDS:** Prostate cancer, androgen receptor, circulating tumor cells, disseminated tumor cells, enzalutamide, abiraterone, metastasis

3. **OVERALL PROJECT SUMMARY:**
   ACCOMPLISHMENTS
   Aim 1 Determine whether AR variants and the associated mitotic transcriptome can be isolated from blood spiked with AR variant transfected LNCaP and blood samples from men with CRPC after treatment with abiraterone.

   1a. Determine if splice variant AR and its transcriptome can be detected using CTC isolation. Peripheral blood is spiked with 0, 1, 5, 50, 100, 500 and 1000 LnCaP transfected with ARV567ES, or ARV7 per 3.5 ml of blood. Controls are untransfected LNCaP. Methods used are processing of samples by Rarecyte, collection of purified LNCaP and extraction for RNA to perform qRT-PCR for splice variants and variant transcriptome.

   Results:
   As described in the annual report, the Rarecyte assay originally proposed for this project did not reproducibly isolate CTC below 1000 cells/3.5 ml of blood. Transcripts from successful isolations did not match between duplicates and this assay for isolation of CTC was abandoned. As an alternative approach, we utilized the AdnaTest (Adnagen) assay as

modified by Antonarakis et al (1). This assay reliably isolated splice variant transcript as detected by QT-PCR from as low as 5 spiked LNCaP 95 cells overexpressing AR V7 splice variants in 5 ml of blood (spiking experiment utilized 0, 5, 10 and 50 cells in female whole blood). Control experiments utilizing LNCAP alone vs. ARsv expressing LNCaP 95 were used and the assay demonstrated the ability to detect overexpression of UBE2C in the variant transfectant cells compared to controls (data not shown).

1b. Determine if CTC from abiraterone resistant prostate cancers contain splice variant AR and mitotic transcriptome.

Aim 2. Determine whether AR variants and the associated mitotic transcriptome can be isolated from DTC acquired from men with metastatic CRPC after abiraterone therapy.

Results:
As noted above, in spiking experiments using 50 LNCaP 95 cells expressing ARV7, we were able to detect overexpression of UBE2C mRNA, which is a transcript upregulated by majority of ARsv, including ARV7, ARv567es and ARv5es. Attempts to delineate upregulation of the other transcripts defined from ARV7 overexpressing cells from both CTC and DTC analysis could not be isolated due to limiting amounts of cDNA.

When it was apparent that it would be difficult to isolate relevant transcripts, we addressed a second issue, namely whether the presence of ARsv in CTC is correlated with ARsv isolated from tumor biopsies. We have an active program performing metastasis biopsy and were the leading site for biopsy acquisition in the SU2C/PCF International "Dream team" CRPC biopsy effort (https://www.standup2cancer.org/dream_teams/view/precision_therapy_for_advanced_prostate_cancer)

As noted in the previous report, investigators from our team interrogated the metastasis biopsy RNA seq data from SU2C biopsies from all sites and identified multiple AR splice variants which had not been detected in clinical biopsy specimens (see Figures 1 and 2). The manuscript including this data was published in *Cell* (Robinson et al., 2015) since the time of the previous report on this project.

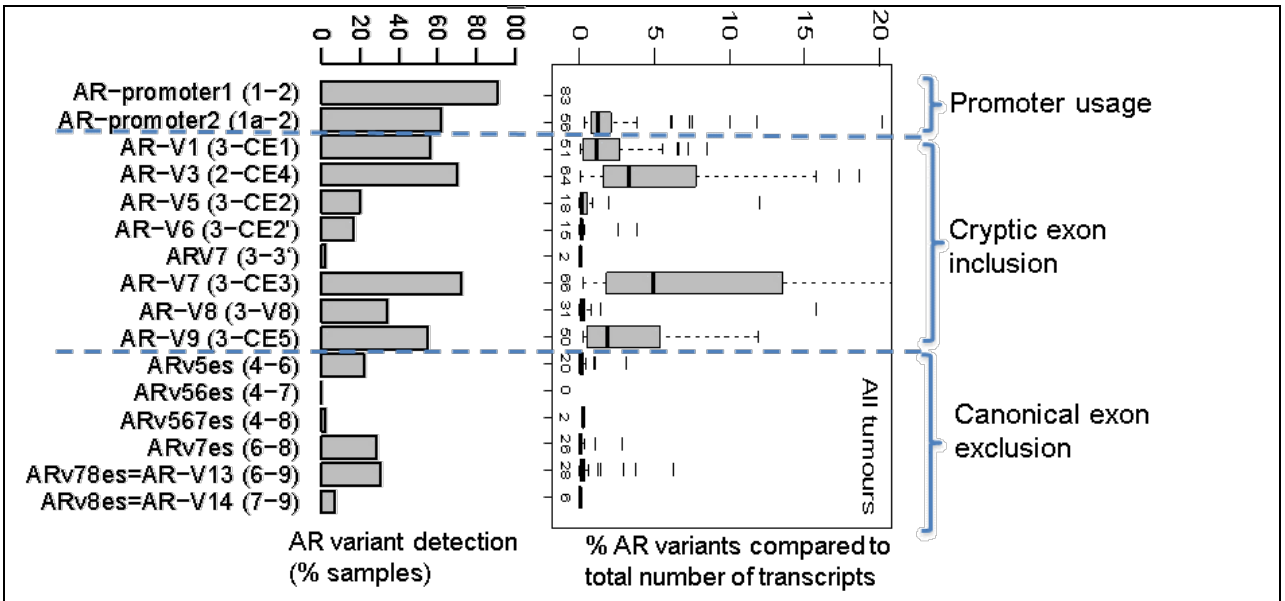


Figure 1 - transcripts from 94 biopsies acquired from patients in the SU2C cohort were analyzed for the presence of ARsv. Samples were analyzed for use of specific promoters, presence of cryptic exons or exclusion of exons present in full length receptor (exon skipping variants). 73 patient samples (78%) contained at least one ARsv and 69 (73%) contained more than one ARsv.

Because these unique ARsv isoforms were more common than anticipated and might have the ability to activate a similar pathway to ARV7 and ARv567es, the Plymate laboratory generated constructs of these ARsv and determined that the novel exon skipping splice variant ARV5es is autonomously transcriptionally active, is resistant to suppression by the AR antagonist enzalutamide and is localized to the nucleus in the absence of ligand, supporting its proposed role as a ARsv which is autonomously active independent of ligand (Figures 3-5, Appendix 1).

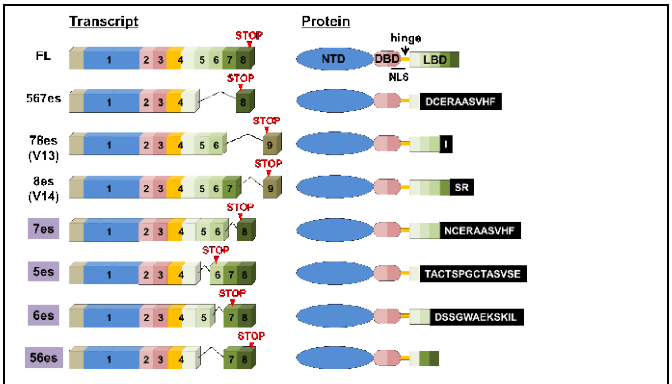


Figure 2. Schematic representation of AR-FL and AR-Vs Note that ARv5es, v6es, v56es, and v7es are newly discovered AR-Vs through construction of splicing landscapes of AR in mCRPC (Robinson et al., 2015).

This work is in preparation for submission.

We then collected CTC from peripheral blood, and disseminated tumor cells (DTC) from bone marrow aspirate from twenty six patients undergoing metastasis biopsy at our site for other studies. All patients were initiating or finishing therapy with abiraterone or enzalutamide. Nineteen of twenty six patients had previously been treated with abiraterone and six were resistant to both abiraterone and enzalutamide at the time of sample acquisition.

CTC were evaluated by the Plymate lab using Adnatest with ARsv products isolated using RT-PCR and agarose separation of the products with subsequent sequencing of the bands. Metastasis

biopsies performed at the same time underwent RT-PCR, agarose separation and sequencing as for the CTC. DTC were isolated from marrow aspirates from bone procured at the time of metastasis biopsy by the Morrissey lab as previously described (Morgan et al., 2009) The CTC, DTC and tumor ARsv sequencing results are included in Table 1. We were able to sequence ARsv from metastasis only from a subset of patients (8) as the majority of the patients were undergoing biopsy as a component of other studies (SU2C and the Pharmacodynamic Abiraterone study (NCT01503229)) and all sequencing had to be performed out of the single extra biopsy sample taken. Similarly DTC could be assessed only from 9 patients. All DTC patient samples were PSA positive and 8 were positive for EpCam.

	CTC			DTC			Metastasis		
ID	AR	ARV7	Other ARsv	AR	ARV7	Other ARsv	AR	ARV7	Other ARsv
7	**+**			+	+	**-**	**+**	+	
9				+	-	**-**	**+**		
17	**+**			No results			**+**		5es, 7es
19	**+**						**+**	+	
22	**+**						**+**	+	
23	**+**						**+**	+	567es
25	**+**		56es				**+**	+	56es
26	**+**	+					**+**	+	567es
1	+	+		No results			**No results**		
2	+	+		+	-	**-**			
3				+	-	**-**			
4				+	+	**-**			
5				-	-	**-**			
6				No results					
8									
10				**-**	**-**	**-**			
11	+			+	+	**-**			
14	+			+	-	**-**			
15	+	+		No results					
16									
18	+								
20	+								
21									
24	+		567es						
27	+		5es						
28	+	+							

Table 1 – AR species in CTC, DTC and metastasis biopsy in patients receiving second generation AR targeting agents (abiraterone or enzalutamide). "AR" reflects detection of full length androgen receptor

Although limited by the limited number of metastases available for sequencing, Table 1 demonstrates that;

1. Fewer AR species are detected in the Adnatest assay in our laboratory when compared to either DTC or metastasis biopsy in this cohort.
2. ARV7 and exon skipping variants (ARv567es, AR5es, AR56es, AR7es) can be detected by CTC analysis or metastasis sequencing although metastasis sampling detects a larger number of ARsv.
3. The frequency of ARV7 positive CTC on Adnatest in this cohort is lower than originally reported in patients initiating abiraterone or enzalutamide (Antonarakis et al). In the original study, 55% of patients who had received abiraterone had detectable ARV7 transcripts, whereas in this study 4 out of the 22 patients treated with abiraterone had detectable ARV7 (18%)
4. DTC were technically challenging to collect in patients undergoing metastasis biopsy, likely reflecting the timing of DTC acquisition after metastasis biopsy
5. ARsv were commonly found in the small cohort of patients (50%) for whom metastasis sequencing was available, including the novel ARsv originally described in the CRPC 150 cohort of the SU2C paper (Robinson et al, Cell).

This is a pilot study and so drawing conclusions based on the small number of patients sampled is premature. Determining whether the presence of ARsv in CTC or in biopsy is more reflective of the relevant amount of ARsv important for resistance will require correlation of ARsv species in CTC and tumor biopsy with outcome data from the SU2C cohort. Because the patient data in the current study was de-identified, it is not possible to assess the subsequent response or resistance to therapy based on the presence of ARsv. Despite these caveats, it is of interest that the original report of ARV7 in patients starting abiraterone or enzalutamide documented a relatively high frequency of ARV7 positive CTC, was not borne out in this group of patients pretreated with abiraterone. It is plausible that the data from Antonarakis et al reflects patients with a larger amount of ARV7 in their tumor and therefore more positive ARV7 CTC.

Future work in this area will be dependent on having access to a more extensive cohort of patients with both CTC and metastasis biopsy ARsv. We are acquiring access to the SU2C biopsy ARsv data to pair with the CTC data from the 18 patients who were unable to be sequenced from the single metastasis biopsy available. Because our collaborators (Bradley and Dvinge) were able to perform this type of analysis from the original 150 patients in the SU2C/PCF cohort, we believe it highly likely that we will be able to perform this correlation in a significantly larger number of samples beyond the 8 patients for whom metastasis sequencing is available at present. In addition, we are co-investigators in the ongoing DOD project coordinated by Dr. Jun Luo of Johns Hopkins assessing the reproducibility of the Adnatest CTC assay and the frequency of ARV7 in a much larger cohort of patients (~ 600).

4. **KEY RESEARCH ACCOMPLISHMENTS:**

- Detection of novel exon skipping ARsv from CTC and metastasis biopsies
- Characterization of the autonomous AR transcriptional activity of ARv5es with publication of that work pending
- Preliminary observations that CTC collection via Adnatest does not detect the same level of ARV7 previously reported

- Preliminary observations that metastasis provides a more comprehensive sampling of underlying mechanisms of resistance
- Pending correlation of these CTC results with the SU2C tumor biopsy analysis for ARsv from the same patients
- Collaboration with Johns Hopkins and Royal Marsden in the ongoing "Non-invasive detection of AR-FL/AR-V7 as a predictive biomarker for therapeutic resistance in men with metastatic castration resistant prostate cancer" being supported by DOD.

REFERENCES:

Antonarakis, E. S., Lu, C., Luber, B., Wang, H., Chen, Y., Nakazawa, M., Nadal, R., Paller, C. J., Denmeade, S. R., Carducci, M. A., *et al.* (2015). Androgen Receptor Splice Variant 7 and Efficacy of Taxane Chemotherapy in Patients With Metastatic Castration-Resistant Prostate Cancer. JAMA Oncol *1*, 582-591.

Morgan, T. M., Lange, P. H., Porter, M. P., Lin, D. W., Ellis, W. J., Gallaher, I. S., and Vessella, R. L. (2009). Disseminated tumor cells in prostate cancer patients after radical prostatectomy and without evidence of disease predicts biochemical recurrence. Clin Cancer Res *15*, 677-683.

Robinson, D., Van Allen, E. M., Wu, Y. M., Schultz, N., Lonigro, R. J., Mosquera, J. M., Montgomery, B., Taplin, M. E., Pritchard, C. C., Attard, G., *et al.* (2015). Integrative clinical genomics of advanced prostate cancer. Cell *161*, 1215-1228.

5. **OPPORTUNITIES FOR TRAINING AND PROFESSIONAL DEVELOPMENT:** Nothing to report

6. **PLANS DURING THE NEXT REPORTING PERIOD:** Final report

7. **IMPACT:**

Impact on the principle discipline: The importance of the current project is the demonstration that relevant markers of resistance such as AR splice variants can be detected in multiple types of tissue. The study shows that metastasis biopsy is a more sensitive test for the presence of ARsv which may potentially play a role in resistance to abiraterone and enzalutamide. Work to correlate metastasis biopsy ARsv with treatment resistance is ongoing.

Impact on the other disciplines: None

Impact on technology transfer: None

Impact on society: The current project is a study of how to detect markers of resistance to therapy for prostate cancer. Work that improves the ability to appropriately determine the right therapy for patients dealing with cancer both improves therapy for the patient and limits costs to society. Both of these outcomes have a positive impact on society.

8. **CHANGES/PROBLEMS:**

Per the summary discussion above, the focus of the project changed and was approved at the annual report which was necessary due to delay in initiating the study. No additional changes to report.
There were no changes on expenditures, human subjects, vertebrate animals, biohazards.

9. **PRODUCTS**

a. **PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS:**

Tu et al, Characterization of exon skipping androgen receptor splice variants (in preparation)

Montgomery et al. Correlation of the circulating markers of androgen receptor splice variants with tumor expression (in preparation)

b. **INVENTIONS, PATENTS AND LICENSES**: None

10. **PARTICIPANTS**

NAME	ROBERT MONTGOMERY
PROJECT ROLE	PRINCIPAL INVESTIGATOR
RESEARCHER IDENTIFIER	0000-0003-4459-0295
NEAREST PERSON MONTH WORKED	2
CONTRIBUTION TO PROJECT	Dr. Montgomery coordinated all aspects of the project
FUNDING SUPPORT	None other than this grant

NAME	COLM MORRISSEY
PROJECT ROLE	CO-INVESTIGATOR
RESEARCHER IDENTIFIER	0000-0003-1906-5333
NEAREST PERSON MONTH WORKED	1
CONTRIBUTION TO PROJECT	Dr. Morrissey coordinated performance of the DTC separation and sequencing in his laboratory
FUNDING SUPPORT	None other than this grant

NAME	STEPHEN PLYMATE
PROJECT ROLE	CO-INVESTIGATOR
RESEARCHER IDENTIFIER	NO ORCID ID
NEAREST PERSON MONTH WORKED	0
CONTRIBUTION TO PROJECT	Dr. Plymate coordinated performance of the Adnatest assay in his laboratory.
FUNDING SUPPORT	None other than this grant

NAME	SANDY LARSON
PROJECT ROLE	PRINCIPAL INVESTIGATOR
RESEARCHER IDENTIFIER	NO ORCID ID
NEAREST PERSON MONTH WORKED	3
CONTRIBUTION TO PROJECT	Sandy Larson isolated DTC and sequenced the cells for AR splice variants
FUNDING SUPPORT	None other than this grant

There were no changes in personnel or support during the study period. Nothing to report

11. **REPORTABLE OUTCOMES:** None

12. **OTHER ACHIEVEMENTS:** None

13. **APPENDICES:**

    Appendix 1 – Figures 3-5

Supplementary data

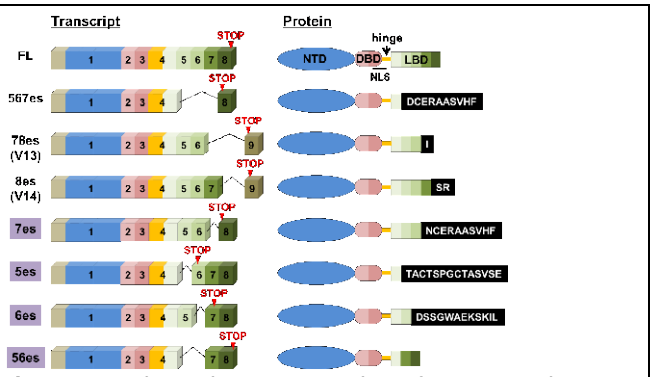


Figure 2. Schematic representation of AR-FL and AR-Vs Note that ARv5es, v6es, v56es, and v7es are newly discovered AR-Vs through construction of splicing landscapes of AR in mCRPC (Robinson et al., 2015).

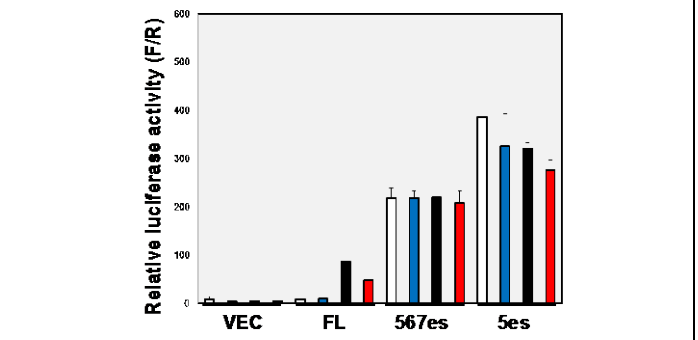
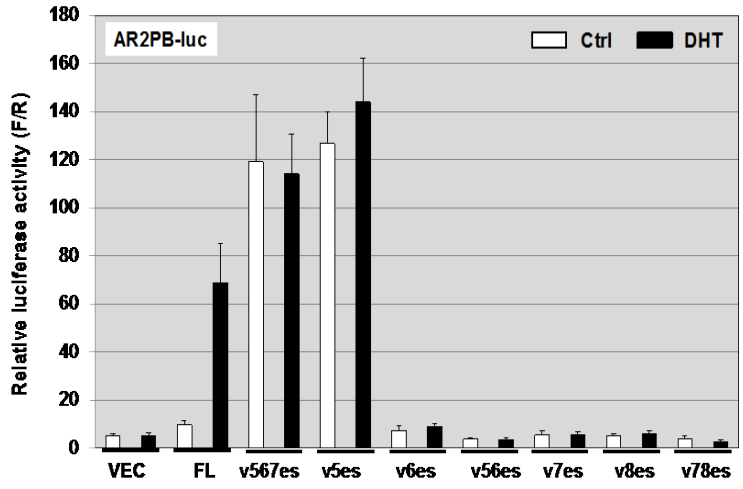
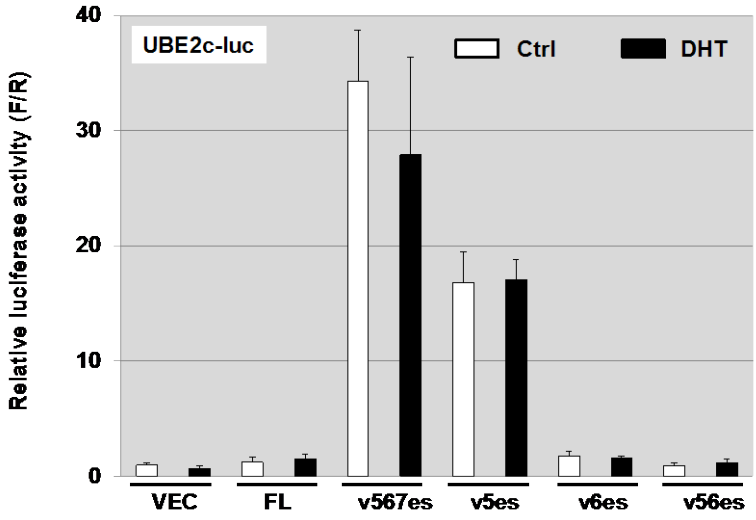


Figure 4. ARv5es is resistant to enzalutamide Androgen-depleted M12 cells were triply transfected with AR-expression vector encoding AR-FL, ARv5es or v567es with the expression vectors for firefly and *Renilla* luciferase activities. Transfectants were treated in the same manner as described in Fig. 2A except for pre-treatment with 10 µM enzalutamide (DMSO as a vehicle control) 2 h prior to DHT treatment. The firefly luciferase activity of each sample was normalized to the *Renilla* luciferase activity. The data represent the mean ± SD of three wells per condition. Similar results were obtained from two independent experiments. *P< 0.0005 by two-tailed t-test.



A.



B.

Figure 3 Transactivation activities of AR-FL and AR-V on the canonical androgen-responsive reporter. PCR from biopsy in one patient revealed the presence of AR^{v5es}. This is a constitutively active AR variant as demonstrated by aprobasin ARE-luciferase reporter activity in M12 cell transfected with a plasmid expressing AR^{v5es} (A.) and the AR-variant UBE2C luciferase promoter assay (B.). This AR variant had previously been reported from AR-seq data by Robinson et al.

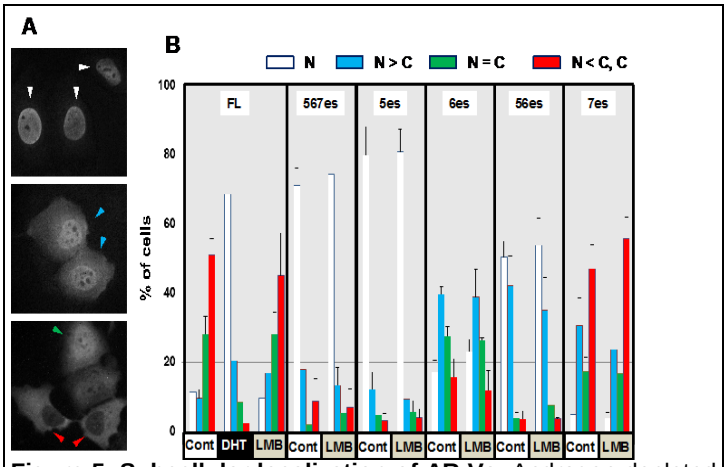


Figure 5. Subcellular localization of AR-Vs. Androgen-depleted M12 were transfected with the expression vectors for FLAG-tagged AR-FL or AR-V. The cells were treated 24 h later with DHT (1 nM), LMB (10 ng/ml), or vehicle control for 4 h. A. The representative immunofluorescence images for ARv5es (top panel), v56es (middle panel), and v6es (bottom panel). B. Subcellular localization of AR-FL and AR-V was determined in approximately 200 cells according to the following criteria. Cells display predominant nuclear FLAG immunofluorescence (white triangles); nuclear > cytosolic staining (blue); nuclear = cytosolic (green); cytoplasmic staining (red).